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Seasonal variations in antioxidant compounds of *Olea europaea* leaves collected from different Italian cultivars

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Summary

The objectives of this research were to study the best conditions for phenol extraction from *Olea europaea* L. leaves and to evaluate the content of phenolics and the antioxidant activity by *in vitro* assays in olive leaves harvested in different periods from four different Italian cultivars. The results showed that leaves/solvent ratio and temperature showed a significant and positive correlation with phenol extraction yield. In all harvesting periods the phenol content of Dolce Agogia samples was higher ($P \leq 0.05$) with respect to Moraiolo, Leccino and Frantoio samples. The results of analysis by high-performance liquid chromatography coupled with diode array detector (HPLC-DAD) showed that Dolce Agogia had the highest concentrations of hydroxytyrosol and oleuropein. Moreover the highest contents of bioactives have been found in olive leaves harvested in December and March. In these months, corresponding to olive harvest and pruning of olive trees, leaves represent a waste product and this interesting result could be useful in the production of nutraceuticals.

Introduction

The olive tree, *Olea europaea* L., is one of the most important fruit tree in Mediterranean countries. The olive fruit, its oil, and the leaves of the olive tree have a rich history for nutritional, medicinal, and ceremonial purposes (GHANBARI et al., 2012).

It is known that natural foods and food-derived antioxidants such as phenolic phytochemicals have interesting biological properties (QUIDEAU et al., 2011). In recent years, interest in olive leaves has grown among scientists because they contain many potentially bioactive compounds with health benefits (EL and KARAKAYA, 2009). Therefore, the demand of whole olive leaf and olive leaf extract has increased for use in food, additives and functional products (LAFKA et al., 2013). Thus, olive leaf extract is now a popular nutraceutical, a dietary supplement manufactured in liquid or capsule form (HAYES et al., 2011; DE BOCK et al., 2013).

The most abundant biophenol in olive leaves is oleuropein (OLE), followed by hydroxytyrosol (HT). Olive leaves have the highest antioxidant and scavenging power among the different parts of the olive tree, in fact OLE content ranges between 1% and 14% (JAPÓN-LUJÁN et al., 2006). Accordingly, *in vitro* and *in vivo* studies have documented the antioxidant, hypolipidaemic, and especially hypotensive, anticancer and cardioprotective properties of OLE (HASSEN et al., 2014). Also the HT shows interesting biological properties (HU et al., 2014).

The chemical composition of olive leaves varies depending on several conditions such as origin, proportion of branches on the tree, climatic conditions, storage conditions, and moisture content (SABRY, 2014). Several studies have investigated the phenolic composition of olive leaves (PEREIRA et al., 2007; ABAZA et al., 2011; SALAH et al., 2012; STAMATOPOULOS et al., 2014). Some authors studied the influence of the solvent type on the extraction of phenolic compounds and the antioxidant properties of the extracts obtained from Chétoui olive leaves (ABAZA et al., 2011). Other authors studied the phenolic composition and biological activities of olive leaves from different varieties grown in Tunisia (SALAH et al., 2012). A high content of polyphenol and flavonoid was detected in all varieties and OLE was the major compound. Also, olive leaf extract exhibited a good antioxidant activity. It has been reported that the combination of steam blanching process and multistage extraction (after optimization) is advantageous since it provides short extraction times (≤ 30 min) at moderate operation temperatures (40 °C) (STAMATOPOULOS et al., 2014). Recently, TALHAOUI et al. (2015) analyzed by high performance liquid chromatography-diode array detector-time-of-flight-mass spectrometry (HPLC-DAD-TOF-MS) leaves from six olive cultivars collected at four different times. This research concerns the evaluation of antioxidant fraction in olive leaves from four different Italian cultivars (Dolce Agogia, Moraiolo, Leccino, Frantoio), harvested at four different periods (December 2013; March, June and September 2014). Firstly, experimental design methodology was used to optimize operating conditions of the phenol extraction. Then total phenolic (TP) content and antioxidant properties by two scavenging activity methods, DPPH (2,2-diphenyl-2-picrylhydracyl hydrate) and ABTS [(2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], have been determined on olive leaf extracts. Moreover, to quantify OLE and HT a chromatographic method, HPLC with diode array detector (DAD), was developed. This study deepens the knowledge on antioxidant compounds of olive leaves harvested in different periods from some characteristic olive cultivars of central Italy, also for the purpose to highlight the best period of the year to harvest olive leaves for using as source of bioactives.

Materials and methods

Olive leaf samples

Olive leaves from four different *Olea europaea* L. cultivars (Dolce Agogia, Moraiolo, Leccino and Frantoio) were collected from an olive orchard located in the area of Perugia (Umbria, central Italy). The selected cultivars, grown under the same agronomic and environmental conditions, were some of the most widespread cultivars in central Italy. The trees had not been irrigated and no phytosanitary treatments had been applied in the last year. Leaves were randomly harvested in different times (December 2013; March, June and September 2014) from five trees of each cultivar. Branches with both young and mature leaves were collected at the operator height around the whole perimeter of each tree. The collected samples were stored in plastic bag until arrival at the laboratory, where they were immediately separated from the branches. Then the leaves were dried in a ventilated oven for 72 h at 40 °C and stored away from light and humidity until extraction.

Extraction and isolation of phenols

The optimization of phenol extraction conditions was carried out by MODDE 5.0 experimental design software (UMETRICS AB, Umeå, Sweden). The following factors were considered: EtOH%,

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leaves/solvent (L:S) ratio and temperature. The solvent composition ranged from 0 to 100 % EtOH in H_2O and the temperature from 20 to 60 °C. The L:S ratio, indicated as 1, 2 and 3, referred respectively to 0.7, 1.7 and 2.7 g of dried leaves in 75 mL of solvent. TP content, determined as reported in a following paragraph and expressed as milligrams of gallic acid equivalent (mg GAE) in total extract, was selected as response. D-optimal design including three replicated center points was employed and a total of 14 experiments was obtained. The extractions were conducted in random order using the experimental conditions indicated in the worksheet (Tab. 1). The model was then fitted using multiple linear regression analysis. 2.7 g of dried leaves from each tree were added with 75 mL of a mixture H_2O/EtOH (40:60, v/v) and homogenized using a homogenizer (Oster, model 869-50R, USA). After that the mixture was kept under magnetic stirring for 30 min at 60 °C and filtered through a Whatman filter paper in a Büchner funnel. All extractions were carried out in duplicate. The obtained extracts were subjected to the analytical determinations, reported in the following paragraphs.

Determination of phenolic compounds

The TP content was determined spectrophotometrically according to the method of SINGLETON and ROSSI (1965) and expressed as milligrams of gallic acid equivalent per gram (mg GAE/g) of dried leaves. A calibration curve was built using gallic acid as standard (97.5-102.5 % titration, Sigma, St. Louis, MO, USA), in the range 0.0025-0.0125 mg/mL. 0.5 mL of Folin-Ciocalteu reagent (Sigma), 2.0 mL of 20 % Na_2CO_3 and 6.5 mL of deionized H_2O were added to leaf diluted extract (1.0 mL). The solution was mixed, incubated in the dark for 90 min and then the absorbance was measured at 750 nm. All determinations were carried out in duplicate.

DPPH assay

The DPPH free radical-scavenging activity of olive leaf extracts was determined according to the method described by ASSIMOPOULOU et al. (2005) with some modifications. In brief, a 0.06 mmol/L solution of DPPH$^{\bullet}$ (Sigma) in EtOH was prepared and left for 1 h in the dark at 4 °C. An aliquot of each extract (0.1 mL) was added to 3.9 mL of DPPH$^{\bullet}$ solution and vortexed. The samples were left for 30 min in the dark, and then their absorbance was measured at 517 nm.

The percentage of antioxidant activity (AA %) was calculated using the following formula:

$$AA\% = (Abs_c - Abs_s / Abs_c) \times 100$$

where:

Abs_c is the absorbance of the control solution (containing only DPPH$^{\bullet}$)

Abs_s is the absorbance of the DPPH$^{\bullet}$ solution mixed with sample.

All determinations were carried out in duplicate.

ABTS assay

The ABTS assay was determined by earlier reported method (TAWAHA et al., 2007) with slight modifications. ABTS radical cation (ABTS$^{\bullet+}$, Sigma) was produced by reacting ABTS solution with $K_2S_2O_8$. An aqueous solution of 7 mmol/L ABTS was prepared and used to dissolve 2.45 mmol/L $K_2S_2O_8$. The mixture was left in the dark at room temperature for 12-16 h and then it was diluted with H_2O until the absorbance reached 0.70 ± 0.02 at 734 nm. After addition of 4 mL of diluted ABTS$^{\bullet+}$ solution to 60 µL of each extract appropriately diluted, the reaction was left in the dark at room temperature for 6 min. After that the absorbance was measured at 734 nm. The antioxidant capacity of each sample was expressed as milligrams Trolox equivalents per gram (mg TE/g) of dried leaves. The calibration line was built using (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid standard (Trolox, 97 %, Sigma) at different concentrations (from 0.1 to 0.5 mg/mL). All determinations were carried out in duplicate.

Instrumentations

Spectrophotometric measurements to determine total phenolic content and antioxidant activity were performed using a Jasco 7850 UV-Vis spectrophotometer (Jasco Inc., Easton, MD, USA). HPLC analysis of olive leaf phenols was performed using a Shimadzu GT-154 system equipped with a Thermo Spectra Series pump, an Agilent Zorbax ODS column (5 µm particle size, 3.0 × 150 mm i.d., Agilent

Tab. 1: Worksheet and response for D-optimal design

Exp. no.	Run order	Factors			Response
		EtOH%	L:S ratio	Temperature (°C)	TP (mg GAE)
1	12	0	1	20	31.7
2	6	100	1	20	17.0
3	3	40	2	20	88.3
4	2	0	3	20	76.1
5	1	100	3	20	42.6
6	11	40	1	40	49.7
7	4	100	2	40	40.0
8	8	0	1	60	38.3
9	9	100	1	60	29.6
10	10	0	3	60	110.9
11	13	80	3	60	196.2
12	5	60	2	40	106.7
13	14	60	2	40	11.0
14	7	60	2	40	97.7

[a] L:S ratio (indicated as 1, 2 and 3) referred respectively to 0.7, 1.7 and 2.7 g of dried leaves in 75 mL of solvent.

Technologies Italia, Milano, Italy) and a Spectra System UV6000LP DAD (Thermo Separation Products, San Jose, CA, USA). Detection was performed on-line using the DAD in the wavelength range 200-700 nm. Separation was achieved by a gradient elution, according to Ortega-Garcia and Peragon (2010). The solvents were water (pH adjusted to 3.1 with 2 mL/L acetic acid) (A) and MeOH (B). The samples were analyzed by gradient elution at a flow rate of 0.8 mL/min. The elution conditions were: initial A-B (90:10); in 5 min A-B (70:30); for 5 min A-B (70:30); in 5 min A-B (60:40); in 2.5 min A-B (50:50); in 2.5 min A-B (40:60); in 2.5 min A-B (30:70); in 3.5 min A-B (0:100); for 4 min A-B (0:100). The column was re-stabilized to the initial conditions for 15 min before the next injection. An injection volume of 20 µL was used. The chromatograms were acquired and the data handled using Xcalibur software version 1.2 (Finnigan Corporation, San Jose, CA, USA). Calibration lines were built using OLE (≥ 90%) and HT (≥ 98%) reference compounds (Extrasynthese, Genay Cedex, France) and the least square method was used to calculate the regression equations. All analyses were carried out in duplicate.

Statistical analysis

The results of the chromatographic analysis and the antioxidant assays are expressed as the mean value and standard deviation (SD) of the five samples of each cultivar. Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA) was used for data analysis.

Results and discussion

Optimization of extraction conditions

To study the influence of the selected variables on TP content, an experimental design was carried out by entering three factors (EtOH%, L:S ratio and temperature) and one response (TP, expressed as (mg GAE), by selecting the screening objective and by choosing the D-optimal experimental design. The experimental design reported acceptable values regarding the percentage of the variation of the response explained by the model ($R^2 = 0.85$) and the percentage of the variation of the response predicted by the model ($Q^2 = 0.61$). Fig. 1 shows the coefficient plot of the considered factors (EtOH%, L:S ratio, temperature).

It can be observed that the response was positively influenced by L:S ratio, temperature and their interaction (L:S*temp), while EtOH% influenced to a lesser extent the response and exhibited an inverse correlation. Fig. 2 shows the prediction plots of TP content as a function of the single factors, EtOH% (a), L:S ratio (b) and temperature (c), maintaining fixed the values of the other two factors.

The results confirmed that EtOH% had little influence on the TP content with respect to the other two variables, in fact L:S ratio and temperature showed a significant and positive correlation on extraction yield. In Fig. 3 the two-dimensional contour plot showing the TP content as a function of temperature and L:S ratio, maintaining fixed the percentage of EtOH at 60, is reported.

It can be noted that the highest TP values were obtained when the L:S ratio was 3 (2.7 g of dried leaves in 75 mL of solvent) and the temperature was 60 °C. On the base of the obtained results, the conditions selected for the extraction of olive leaf samples, from different cultivars in different harvesting periods, were the following: ratio 3, 60 °C and 60% EtOH in H_2O. The composition of the extraction mixture was chosen because the extract had a better workability.

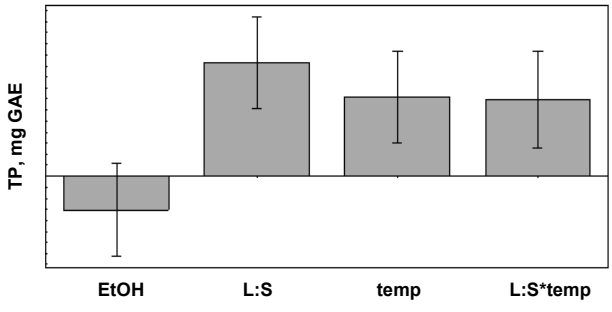


Fig. 1: Coefficient plot of the considered factors (EtOH, ethanol %; L:S, L:S ratio; temp, temperature) for total phenol (TP) response.

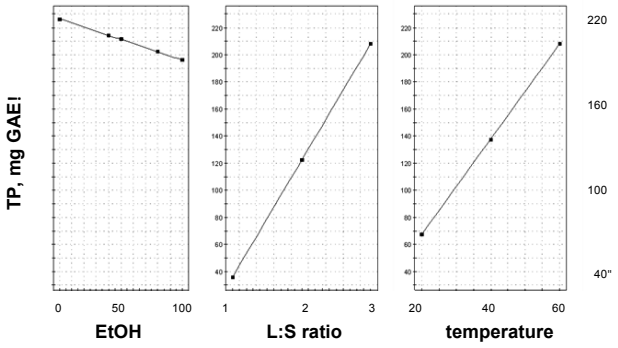


Fig. 2: Prediction plots of the considered factors: EtOH% (L:S ratio 3 and temperature 60 °C); L:S ratio (EtOH 60% and temperature 60 °C); temperature (EtOH 60% and L:S ratio 3) for total phenol (TP) response. L:S ratio values have been described in Tab. 1.

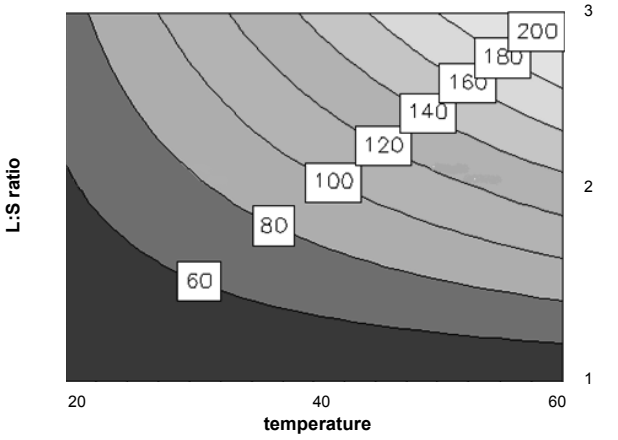


Fig. 3: Two-dimensional contour plot showing the total phenol content as a function of L:S ratio and temperature, when the percentage of EtOH was fixed at 60. L:S ratio values have been described in Tab. 1.

Phenol content and antioxidant activity

Fig. 4 shows the phenol content of all the considered samples determined by Folin-Ciocalteu method.

The mean values of TP in olive leaf extracts ranged from 40.9 mg GAE/g for Frantoio (December harvesting) to 66.6 mg GAE/g for Dolce Agogia (June harvesting). Phenol content increased from December to March ($P \leq 0.01$) and from March to June ($P \leq 0.01$), in fact the highest TP values ($P \leq 0.05$) were found in June for all cultivars. Phenol content showed marked variations with plant growth, in fact probably the phenol storage in the leaves is a time-dependent regulated process, according to the life cycle of olive leaves (phenological phase). This hypothesis has been confirmed by other authors (Brahmi et al. 2012; Brahmi et al. 2015). In the

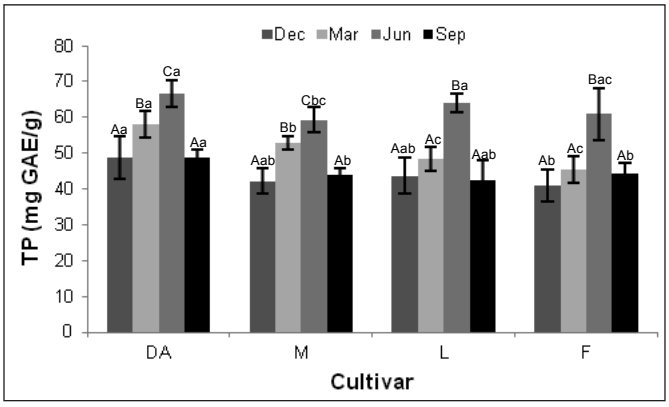
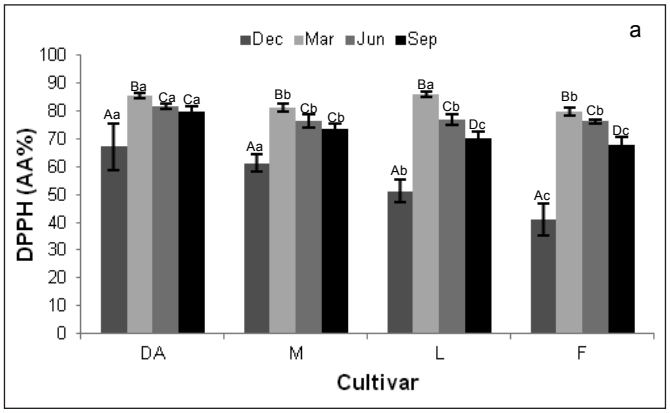


Fig. 4: Total phenol content (mg GAE/g dried leaves, mean values) in Dolce Agogia (DA), Moraiolo (M), Leccino (L), Frantoio (F) olive leaves harvested at December (Dec), March (Mar), June (Jun) and September (Sep). Error bars indicate SD of the means (n = 5). Means with different capital letters indicate significant differences ($P \leq 0.05$) among months within the same olive cultivar. Means with different lower case letters indicate significant differences ($P \leq 0.05$) among olive cultivars within the same month.



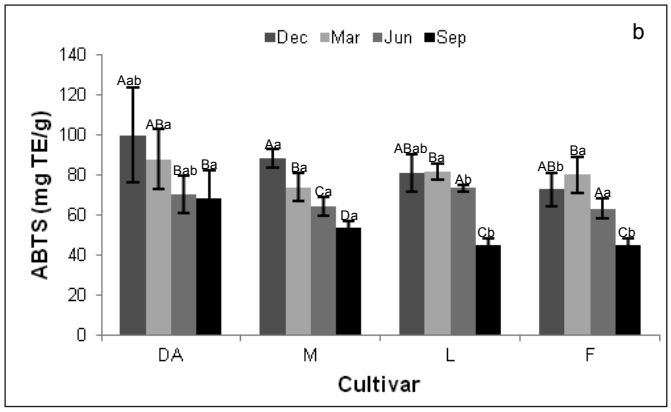


Fig. 5: Antioxidant activity determined by (a) DPPH assay (AA%, mean values) and (b) ABTS assay (mg TE/g, mean values) in olive leaves harvested in different periods from different cultivars. Error bars indicate SD of the means (n = 5). Means with different capital letters indicate significant differences ($P \leq 0.05$) among months within the same olive cultivar. Means with different lower case letters indicate significant differences ($P \leq 0.05$) among olive cultivars within the same month. Abbreviations of olive cultivars and harvesting periods have been reported in Fig. 4.

harvesting period of March the phenol content of Dolce Agogia samples was higher ($P \leq 0.05$) with respect to Moraiolo, Leccino and Frantoio. Results comparable to those presented in this research have been reported by Alzweiri and Al-Hiari (2013) for an olive leaf methanolic extract (40 mg GAE/g). On the contrary, Abaza et al. (2011) reported lower values (from 16.52 to 24.93 mg GAE/g) for the second main variety (Chétoui) cultivated in the north of Tunisia. Salah et al. (2012) reported instead higher values (from 73.05 of Sevillane to 144.19 mg GAE/g of Limouni variety) for different Tunisian varieties in respect to those reported in this study. Ahmad-Qasem et al. (2014) studied the phenol content of Spanish olive leaves (variety Serrana) dried with different methods and they found significant differences. In fact they reported the highest values (59.0 mg GAE/g) for samples dried using hot air and the lowest (36.3 mg GAE/g) for freeze dried samples.

In order to obtain data about the antioxidant capacity of olive leaf extracts, two different *in vitro* assays, DPPH and ABTS, have been performed. Free-radical scavenging potential of samples was first tested by DPPH assay and the results are shown in Fig. 5a.

Generally, it can be observed that all olive leaf extracts exhibited high radical scavenging activity even if it is well known that antioxidant capacity is influenced by several factors, among which harvesting period and cultivar (Brahmi et al., 2015). A similar trend was obtained for each cultivar, according to the biological cycle of olive leaves, in fact antioxidant activity was the highest in March, when the leaves had completed their growth, while it decreased slightly or strongly ($P \leq 0.05$) until September, when started the ripening of the fruit. The lowest values had been found for all cultivar samples harvested in December (from 40.9 of Frantoio to 67.1 % of Dolce Agogia), while olive leaves from Leccino cultivar harvested in March exhibited the highest activity (86.1 %). The results reported by Abaza et al. (2011) showed that the DPPH radical scavenging activity increased with the extract concentration (at 0.5 mg/mL the activity was 59.74 %, using 70 % EtOH). Also Lafka et al. (2013) studied the influence of the extraction solvent on olive leaf DPPH antioxidant activity and reported the lowest value (18.8 %) for *n*-propanol and the highest (55.0 %) for EtOH. Fig. 5b shows the results of the radical scavenging potential of samples tested by ABTS assay. The values of antioxidant activity ranged from 44.8 mg TE/g (179.0 µmol TE/g) for Leccino leaves harvested in September to 99.8 mg TE/g (398.7 µmol TE/g) of Dolce Agogia leaves harvested in December. Moraiolo samples showed a significant decrease ($P \leq 0.05$) from December to March, to June, to September and the values ranged from 88.4 to 53.6 mg TE/g. With regard to Dolce Agogia samples, significant differences between December and June and between December and September ($P \leq 0.05$) have been found. As regards Leccino and Moraiolo cultivar significant differences ($P \leq 0.05$) had also been observed between December and September, March and June, March and September, June and September. The antioxidant activity determined by ABTS showed significant differences also between Moraiolo and Frantoio and between Moraiolo and Leccino ($P \leq 0.05$) with regard to September harvest. Not always the trend of ABTS antioxidant activity was similar to that observed for DPPH results and this occurrence could be due to the different qualitative composition of phenols in the leaves, as already suggested by Brahmi et al. (2013). The values of antioxidant activity determined by ABTS assay were lower than those reported by Abaza et al. (2011) which found values in the range 629.87-1064.25 µmol TE/g. Even higher values for antioxidant activity (1.80-4.36 mmol TE/g) have been reported by Altiok et al. (2008) which studied the isolation of polyphenols from the extracts of olive leaves by adsorption on silk fibroin. On the contrary Ahmad-Qasem et al. (2014) reported very lower values (4.5-7.5 mg TE/g) for ABTS assay than those reported in this research.

HPLC-DAD analysis

Among the compounds detected by HPLC-DAD particular attention has been given to HT and OLE, the main phenol compounds in olive leaves. Fig. 6a shows the content of HT in olive leaf extracts for the considered samples.

The HT content varied considerably considering both the cultivars and the harvesting periods. The values ranged from 1.5 mg/g of Leccino in September to 7.0 mg/g of Dolce Agogia in December. In all considered cultivars the content was higher in December than in June and September ($P \leq 0.05$) and it was also higher in March than in June. It was observed a significant difference ($P \leq 0.05$) of HT content between December and March in Dolce Agogia and between June and September in Leccino samples. Generally, TALHAOUI et al. (2015) reported lower values for HT content (sum isomers), comparable only with Leccino values. Fig. 6b shows the OLE content in the considered samples and the results showed that it was very different among cultivars and harvesting periods. Dolce Agogia exhibited the highest values in each period, while the lowest was found in Frantoio cultivar. In December the OLE content significantly decreased ($P \leq 0.05$) from Dolce Agogia (91.8 mg/g) to Frantoio (9.7 mg/g). The same trend was showed in September from Dolce Agogia (80.2 mg/g) to Frantoio (2.8 mg/g). SAVOURNIN et al. (2001) studied the OLE content in leaves from 14 different olive cultivars and reported values ranging from 9.04 of Aglandau to 13.66% of Picholine cultivar and their data were comparable to that reported in this research. In another study instead, TAYOUB et al. (2012) reported lower values for Syrian varieties harvested at spring and fall. The OLE concentration, indeed, ranged between 5.6 and 9.2 mg/g in spring samples and the highest content was found in Jlott variety. In fall leaf samples the concentration ranged between 4.3 to 8.2 mg/g and the highest content was found in the same variety. Generally, TALHAOUI et al. (2015) reported lower values of OLE content (sum isomers) for six Spanish cultivars.

The results obtained for olive leaf samples have been elaborated in order to highlight possible correlations between the antioxidant activity measured by *in vitro* assays and HT and OLE content. As an example, Fig. 7 shows the significant positive correlations obtained for OLE content and DPPH value, in all the considered harvesting periods. Moreover, for samples harvested in September it was observed that HT content well correlated with DPPH value (R^2 = 0.8354), ABTS value (R^2 = 0.8079) and OLE content (R^2 = 0.9422). In conclusion, in this study the results of phenolic content and

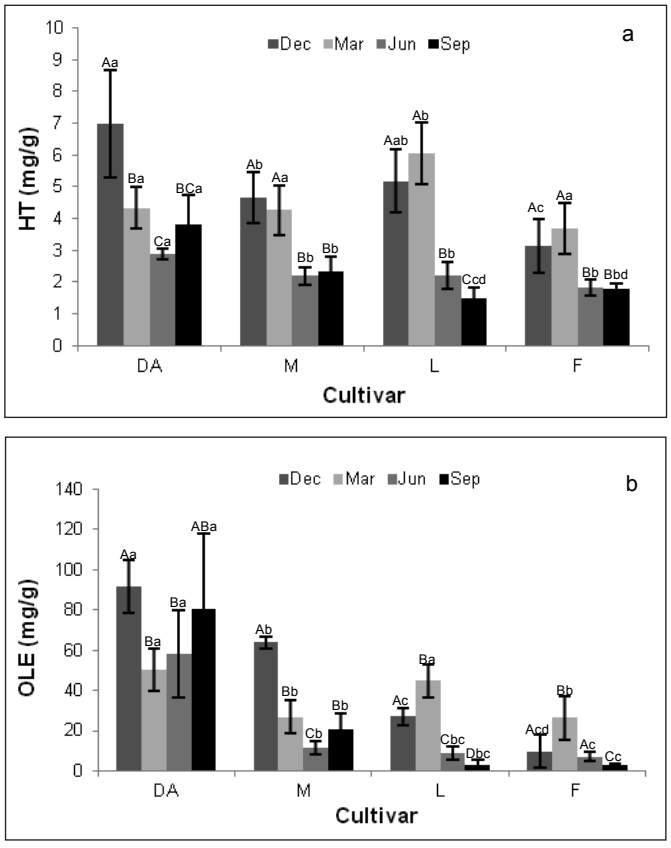


Fig. 6: Hydroxytyrosol (a) and oleuropein (b) contents (mg/g, mean values) in olive leaves harvested in different periods from different cultivars. Error bars indicate SD of the means (n = 5). Means with different capital letters indicate significant differences ($P \leq 0.05$) among months within the same olive cultivar. Means with different lower case letters indicate significant differences ($P \leq 0.05$) among olive cultivars within the same month. Abbreviations of olive cultivars and harvesting periods have been reported in Fig. 4.

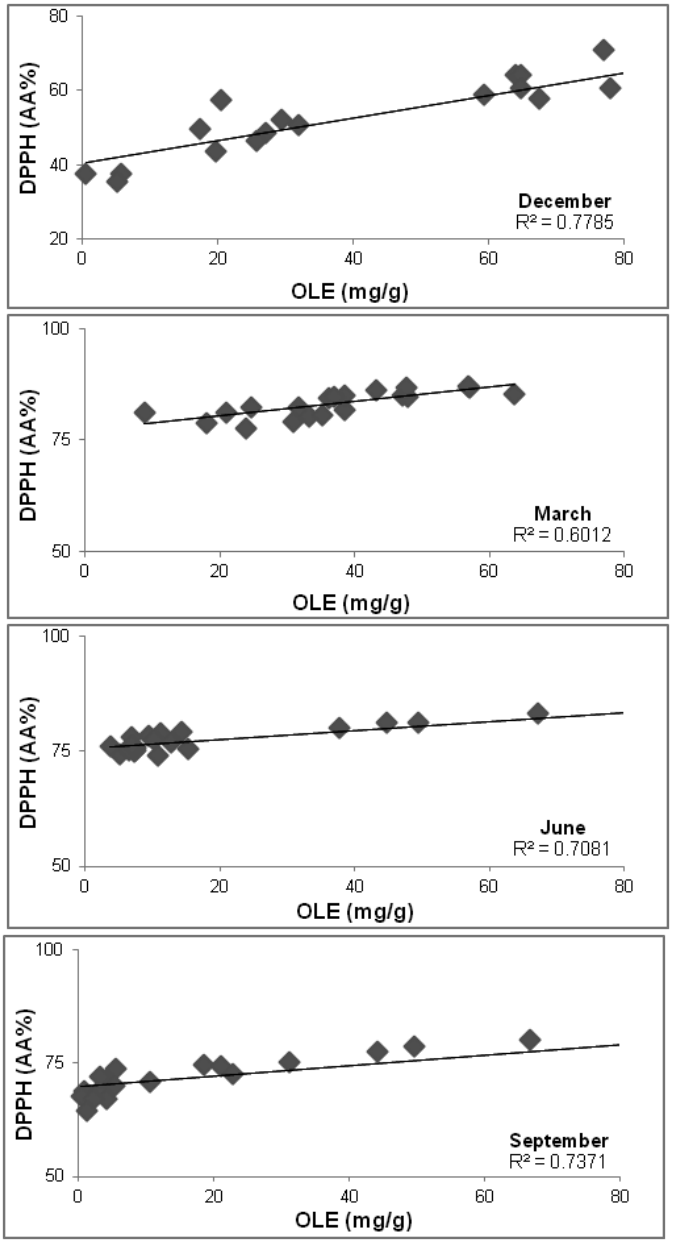


Fig. 7: Correlation plots between oleuropein content (mg/g) and DPPH value (AA%) of olive leaf samples from the considered cultivars harvested in different periods.

antioxidant activity of olive leaves, from different cultivars harvested in different times of year, have been reported. Significant differences in HT and OLE contents among the cultivars have been observed. Dolce Agogia showed the highest values, while Frantoio the lowest ones. In December and March the highest contents of bioactive compounds were found and this result was interesting because olive leaves represent a waste product in these periods, corresponding to olive harvest and pruning of olive trees. The results of this research give information on the optimization of olive leaf phenol extraction useful in the production of nutraceuticals.

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